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Haplotype diversity of mountain hare mtDNA among native mountain hares and introduced brown hares in Scandinavia

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Natural hybridisation and subsequent introgression mediate the transmission of mitochondrial DNA (mtDNA) from native mountain hares (*Lepus timidus*) to introduced brown hares (*L. europaeus*). We investigated mtDNA Restriction Fragment Length Polymorphism among 62 Scandinavian mountain hares, 20 brown hares with mountain hare mtDNA and 19 presumed hybrids from 57 localities in Sweden and Norway. A high level of mtDNA haplotype diversity was detected (0.90 ± 0.026). Mountain hare mtDNA haplotypes transferred to brown hares were different from those among mountain hares, both sympatric and allopatric ($p < 0.05$). One possible explanation is that hybridisation and introgression were more common during the initial phase of contact between the species following the introduction of brown hares, after which some haplotypes have become extinct in declining mountain hare populations, but have been preserved among the brown hares with mountain hare mtDNA.

Introduction

Hare specimens with morphological characters that are intermediate between the brown hare (*Lepus europaeus*) and the mountain hare (*L. timidus*) have long been regarded as hybrids between the two species (Nilsson 1820, Lönnberg 1905, Fraguglione 1959, Gustavsson 1971, Schröder *et al*. 1987). Although hybrids are easy to produce in captivity (Gustavsson & Sundt 1965), the extent and effects of natural hybridisation remained unclear. In a study of species-diagnostic immunoglobulin markers among wild hares of both species in Finland, Schröder *et al*. (1987) failed to detect any hybrids and concluded that if hybridisation occurs, it is rare. The first documentation of gene flow over the species barrier was provided when mountain hare mitochondrial DNA (mtDNA) was detected among wild brown hares in central Sweden (Thulin *et al*. 1997a), where the two species currently occur in sympatry. Thulin *et al*. (1997a) suggested that mountain hare females hybridise with brown hare males and that fertile hybrid females backcross to brown hare males. After a succession of directed backcrosses, the mountain hare mtDNA has been transferred to brown hares, resulting in specimens with typical brown hare morphology but with mountain hare mtDNA. These features are shared by approximately 10% of the brown

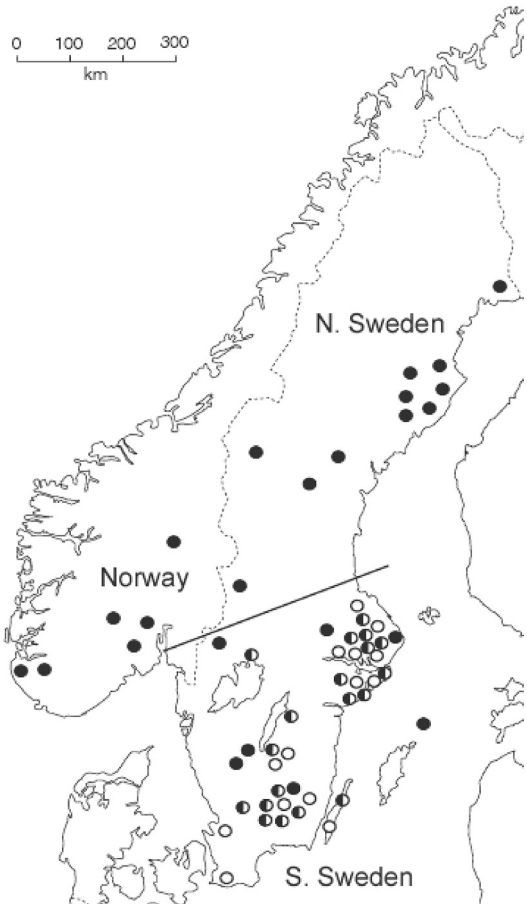

Fig. 1. Sample localities for mountain hares (filled circles) and brown hares (unfilled circles) are shown. The half-filled circles indicate localities where samples from both species and presumed hybrids were collected. The line separates South Sweden, where the two hare species occur in sympatry, and Norway/North Sweden, where only mountain hares occur.

hares in Sweden, locally up to 25% (Thulin & Tegelström 2002). The reciprocal transfer, resulting in mountain hares with brown hare mtDNA, has never been observed.

The brown hare was introduced to Sweden from northern continental Europe (e.g. Denmark, Germany) as a game species during the late 19th century (Lönnberg 1905). It was well established in the southernmost parts of Sweden at the turn of the century (Lönnberg 1908) and later dispersed northwards through additional introductions and natural migration. The present distribution of the brown hare includes most of South Sweden (Mitchell-Jones *et al.* 1999) and still, gradually, expands northwards (Pehrson *et al.* 2002). The mountain hare colonised the Scandinavian Peninsula right after the most recent glacial period. The oldest remains, found in the far south of Sweden, are dated 12 000 years before present (Lepiksaar 1986). When the ice sheet over Scandinavia withdrew, new colonisation routes were created in the northeast, enabling colonisation over Russia and Finland. The current distribution of mountain hares includes all of Scandinavia except the far south of Sweden (Mitchell-Jones *et al.* 1999). The disappearance of mountain hare from the far south of Sweden may be due to interspecific competition with the brown hare, because wherever their distributions overlap the mountain hare seems to diminish or become extinct (Lönnberg 1908, Thulin 2003). After the introduction of brown hares to Sweden, the geographic range of the mountain hares started to shrink (Lönnberg 1908). The number of mountain hares in the game bags from areas where both species occur has decreased considerably during the last decade (Pehrson *et al.* 2002). Hybridisation between native and invasive species is a previously documented effect of species introductions, often with a negative outcome for the native species (Ebenhard 1988, Simberloff 1996). Thus, hybridisation may be important for the apparent competition between the hares in Sweden (cf. Thulin & Tegelström 2002).

In the present study, we hypothesised that interspecies transfer of mtDNA is recent and/or current, and that the mountain hare mtDNA haplotypes detected among presumed hybrids and brown hares therefore should be similar to those carried by sympatric mountain hares. To test this, we investigated variation of mountain hare mtDNA among presumed first generation hybrids and in brown hares, and compared it with the variation of mtDNA among mountain hares that were either sympatric or allopatric to brown hares.

Material and methods

Tissues from hares (kidney, heart and muscle) were collected by hunters at 57 localities in Scandinavia (Fig. 1) during the period 1988–1999. A total of 101 hares were chosen for the present

study, including 62 mountain hares with species-specific mtDNA (Lt), 20 brown hares with transferred mountain hare mtDNA (Le*) and 19 presumed hybrids with mountain hare mtDNA (HLt). Species specificity of mtDNA haplotypes was determined with diagnostic restriction enzyme cutting sites in the cytochrome *b* gene as described by Thulin and Tegelström (2002). The presumed hybrids were classified as hybrids by the hunters who provided the samples. This approach to collect natural hybrids was the only feasible way to obtain a substantial sample.

To estimate mtDNA variation, the whole mitochondrial genome was digested with a set of restriction enzymes and analysed for Restriction Fragment Length Polymorphisms (RFLP). Mitochondria were isolated from 0.5–2.0 g of kidney by differential centrifugation (Jones *et al*. 1988) and mtDNA was purified by phenol/chloroform extraction. The 101 samples were cut with a total of seven tetra nucleotide restriction enzymes (*Hae* III, *Hpa* II, *Hinf* I, *Mbo* I, *Sau* 96I, *Rsa* I and *Dde* I) and the obtained restriction fragments were electrophoretically separated in 5% polyacrylamide gels, using lambda DNA digested with *Bgl* I as a size marker. The fragments were visualised by silver staining as described by Tegelström (1986) and each restriction fragment pattern was given a specific capital letter. Thus, each haplotype was given a seven letter composite code and every unique code was given a number (Table 1).

Genetic distance (*d*), haplotype (*h*) and nucleotide (π) diversity were estimated from the fragment data according to formulae given by Nei and Li (1979), Nei and Tajima (1981) and Nei (1987). Standard errors of the genetic distance values were calculated as described by Upholt (1977). The calculations were performed in the program package REAP v. 4.0 (McElroy *et al*. 1992). To test for geographic heterogeneity in mtDNA haplotype distributions we performed a Monte Carlo simulation as described by Roff and Bentzen (1989). The sample of mountain hares were subdivided into two classes, one for mountain hares that are sympatric to brown hares (South Sweden) and one for allopatric mountain hares from Norway and North Sweden (*see* Fig. 1). The distribution of mtDNA haplotypes among the mountain hares was tested against presumed hybrids and brown hares with mountain hare mtDNA, respectively. The simulations were performed with the MONTE program in REAP (McElroy *et al*. 1992).

The estimates of genetic distances were used to construct phylogenetic inferences with the Neighbor-joining method in the computer program package Phylip v. 3.5 (Felsenstein 1993). To compensate for the non-independence of RFLP fragment data (cf. Dowling *et al*. 1996), we performed an alternative bootstrap procedure. First we excluded one of the seven restriction enzyme profiles at a time, and then recalculated the distance estimates in REAP and reconstructed the Neighbor-joining tree with these new distance estimates. We also excluded half the number of fragments, the smaller and the larger respectively, and reconstructed the Neighbor-joining tree with the recalculated distance values. In this way we obtained a total of ten different Neighbor-joining trees that were controlled for positioning of branches and groups of haplotypes.

Results

Among the 101 hares investigated, we detected a total of 49 mtDNA haplotypes, numbered from 1 to 50 (haplotype 23 is missing because it proved to be identical to haplotype 22 after further analysis). Haplotypes were composed of a mean number of 195 restriction fragments each; hence, approximately 5% of the mitochondrial genome was investigated. The most abundant haplotype (number 1) occurred with a frequency of 0.3, while 37 haplotypes were detected in only one individual each (Table 1). Overall haplotype diversity (*h*) was high and estimated at 0.90 ($\pm$ 0.026) and the overall nucleotide diversity (π) was estimated at 0.0047 (Table 2). The genetic distance between the 49 mtDNA haplotypes detected varied from 0.00022 between haplotype 8 and 50 (one restriction fragment difference) to the largest distance of 0.017 between haplotype 43 and 50.

Among the 62 investigated mountain hares, 34 different haplotypes were detected, of which 30 were found only among mountain hares. Overall haplotype diversity was estimated at

Table 1. MtDNA haplotypes, composition, and the number of mountain hares (N_{Lt}), brown hares with mountain hare mtDNA (N_{Le^*}), presumed hybrids (N_{HLt}) and in total (N_{total}) that carried the haplotypes. The seven-letter haplotype composition code represents the fragment profiles obtained with restriction enzymes *Hae* III, *Hpa* II, *Hinf* I, *Mbo* I, *Sau* 96I, *Rsa* I and *Dde* I, respectively. The groups refer to circles in the Neighbor-joining tree (Fig. 2) and geographic origin refers to sample areas (Fig. 1).

Haplotype number	Haplotype composition	N_{Lt}	N_{Le^*}	N_{HLt}	N_{total}	Group	Geographic origin
1	CAAAAAA	19	4	7	30	–	All regions
2	CACAAAA	1	–	–	1	–	N. Sweden
3	CAACAAA	2	–	–	2	–	S. Sweden
4	CAAAADA	5	1	3	9	–	N. & S. Sweden
5	CAFBCAA	1	–	–	1	–	N. Sweden
6	DCDACCB	2	–	–	2	–	Norway
7	BBBABBA	2	–	–	2	I	S. Sweden
8	DDEDEEJ	–	4	–	4	II	S. Sweden
9	EAIACGA	1	–	–	1	–	N. Sweden
10	BBAKBHA	1	–	–	1	I	S. Sweden
11	BBAKBFA	3	–	–	3	I	S. Sweden
12	CAGECAG	1	–	–	1	–	N. Sweden
13	CAAFAAH	1	–	–	1	–	N. Sweden
14	CAIGCAA	1	–	–	1	–	N. Sweden
15	AEAACIB	1	–	–	1	–	N. Sweden
16	FAJHCJA	1	–	–	1	–	N. Sweden
17	GFKJCKB	1	–	–	1	–	N. Sweden
18	HALIDAE	1	–	–	1	III	Norway
19	FAHFCJA	1	–	–	1	–	S. Sweden
20	IAAHCEF	1	–	–	1	–	N. Sweden
21	DFCACCA	1	–	–	1	–	N. Sweden
22	HAMIDAE	2	–	–	2	III	Norway
24	BBAABFA	1	–	–	1	I	S. Sweden
25	JBJKBLI	1	–	–	1	I	S. Sweden
26	CAAAHAB	1	–	–	1	–	S. Sweden
27	BBAKBAA	1	–	–	1	I	S. Sweden
28	IANHFAF	1	–	–	1	–	S. Sweden
29	JBAKBLI	1	–	–	1	I	S. Sweden
30	BBBKBBA	1	–	2	3	I	S. Sweden
31	CAAABAA	–	–	1	1	–	S. Sweden
32	LCAACIB	–	–	1	1	–	S. Sweden
33	CAALAAA	–	3	–	3	–	S. Sweden
34	CAGAAAK	–	1	–	1	–	S. Sweden
35	CAAAAAL	–	–	1	1	–	S. Sweden
36	CAMAAAM	–	1	–	1	–	S. Sweden
37	EAIGCGA	–	1	–	1	–	S. Sweden
38	CAIMCAA	–	1	–	1	–	S. Sweden
39	GFPNCMB	–	–	1	1	–	S. Sweden
40	CAMAAAB	–	1	1	2	–	S. Sweden
41	IAHFCAF	–	1	–	1	–	S. Sweden
42	CAAAADB	–	–	1	1	–	S. Sweden
43	BBRKBHA	1	–	–	1	I	S. Sweden
44	MASHCAF	1	–	–	1	–	S. Sweden
45	CAPAAAA	–	–	1	1	–	S. Sweden
46	DFANCIB	1	1	–	2	–	S. Sweden
47	MASACAF	1	–	–	1	–	S. Sweden
48	JBAKBAI	1	–	–	1	I	S. Sweden
49	CAMOAAA	1	–	–	1	–	S. Sweden
50	DDODEEJ	–	1	–	1	II	S. Sweden

0.90 (± 0.033). The sample of mountain hares was also subdivided according to sympatry or allopatry to brown hares in Sweden. Haplotype diversity among sympatric mountain hares (LtS) was lower than that among the allopatric mountain hares (LtA) (Table 2). Further, the geographic distribution of mtDNA haplotypes was significantly different between these subdivided mountain hare samples ($p < 0.05$).

The presumed hybrids showed ten different haplotypes, six were found only in this class of hares, and a haplotype diversity of 0.85 (± 0.069). There was no significant difference between the mtDNA haplotype distribution among the presumed hybrids and the mountain hares. In the class of brown hares with introgressed mountain hare mtDNA, hereafter called brown hares, 12 haplotypes were detected of which eight were unique to this class. Haplotype diversity was estimated at 0.92 (± 0.039) and, thus, higher than among sympatric mountain hares (Table 2). Surprisingly, there was a significant difference between the mtDNA haplotypes of brown hares and that of (a) mountain hares from all over Scandinavia (Le* vs. Lt, $p < 0.05$), (b) mountain hares in sympatry with brown hares (Le* vs. LtS, $p < 0.01$), (c) mountain hares in allopatry with brown hares (Le* vs. LtA, $p < 0.05$), and (d) presumed hybrids (Le* vs. HLt, $p < 0.05$).

The genetic distances between the 49 haplotypes were used to construct the Neighbor-joining tree based on the complete dataset (Fig. 2). Three groups of mtDNA haplotypes were consistent among the nine Neighbor-joining trees constructed from a reduced data set (marked with circles in Fig. 2). Among these groups, II and III consist of only two haplotypes each. Interestingly, haplotypes 8 and 50 in group II were only detected in five brown hares from three different localities in southern Sweden. Although these haplotypes were the most deviating within our sample, they still differ with about 8% sequence divergence from a typical brown hare mtDNA haplotype (Thulin *et al*. 1997a). The haplotypes 18 and 22 in group III were detected in three mountain hares from Norway. Group I consists of 10 haplotypes detected among 13 mountain hares and one presumed hybrid from South Sweden. The remaining mtDNA haplotypes form a heterogeneous group of 35 mtDNA haplotypes sampled in a total of 79 hares from all classes of hares with mountain hare mtDNA (Table 1).

Discussion

Mountain hare mtDNA haplotype diversity

The mountain hare mtDNA haplotype diversity detected in the present study is similar to that of pure brown hares in Sweden (0.89 ± 0.002, Thulin & Tegelström 2001) and also in accordance with the high estimates that stem from several other small mammals in Scandinavia (cf. Jaarola *et al*. 1999). The relatively high mtDNA haplotype diversity detected among some of the small mammals in Scandinavia is generally assigned to the mixture of specimens with different geographic origin, representing different gene pools, which occurred during colonisation (Jaarola *et al*. 1999). Mountain hares supposedly colonised Scandinavia through repeated immigration waves over two different post-glacial colonisation routes, one from the south and one from the northeast (cf. Bergengren 1969). A bi-directional colonisation of Scandinavia has been verified in genetic studies of other mammals (e.g. Fredga & Nawrin 1977, Tegelström 1987, Taberlet & Bouvet 1994, Jaarola & Tegelström 1995). We believe that the existence of such a genetic sub-structuring of mountain hares finds support in the significant difference of mtDNA haplotype distribution between mountain hares in allopa-

Table 2. The number of individuals investigated ($N_{ind.}$) and haplotypes detected ($N_{haplo.}$) among Swedish mountain hares in allopatry (LtA) and sympatry (LtS) to brown hares. The class Le* is brown hares with mountain hare mtDNA, and HLt is presumed hybrids with mountain hare mtDNA. The estimated haplotype (h) and nucleotide (π) diversity are given for each class and over all classes.

Class	$N_{ind.}$	$N_{haplo.}$	h (SD)	π
LtA	26	18	0.94 (0.03)	0.0053
LtS	36	19	0.86 (0.05)	0.0038
Le*	20	12	0.92 (0.04)	0.0066
HLt	19	10	0.85 (0.07)	0.0028
All classes	101	49	0.90 (0.03)	0.0047

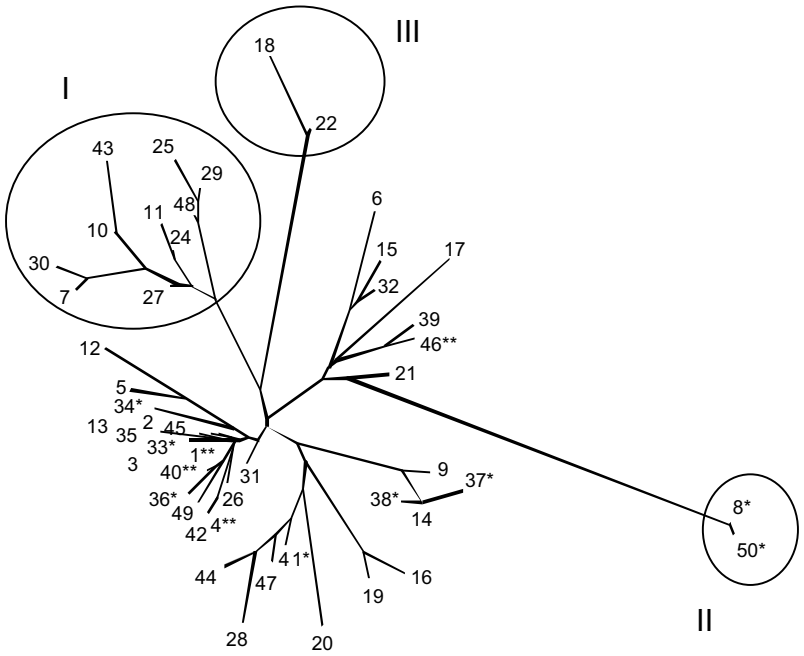

Fig. 2. Neighbor-joining tree constructed from genetic distances between mountain hare haplotypes as estimated from mtDNA restriction fragment data. The circled groups of haplotypes were stable in nine tests, where trees were constructed from distance estimates calculated with different parts of the fragment matrix data excluded. Haplotypes with one asterisk (*) were detected in brown hares only, and two asterisks (**) indicate that the haplotypes were detected in both mountain hares and brown hares.

try and sympatry with brown hares observed in this study. However, more thorough analyses of mitochondrial and nuclear DNA markers are needed to substantiate this hypothesis.

MtDNA of presumed hybrids

The mountain hare mtDNA haplotypes detected among the presumed hybrids did not differ significantly from the mtDNA haplotypes of mountain hares. This is in agreement with our expectations, because if these individuals are the offspring of native mountain hare females and brown hare males, their mtDNA should be similar to what is observed among the sympatric mountain hares. However, a study of autosomal microsatellite DNA variation indicated that only about one third of the presumed hybrids were likely to be true F_1 hybrids (C.-G. Thulin *et al.* unpubl.). The microsatellite data do not enable us to distinguish backcrosses from pure hares or hybrids, and because of this we are unable to sort out any pure hares of either species from our sample of presumed hybrids. Nevertheless, we suspect that some of these presumed hybrids are actually first or second generation backcrosses, or even pure mountain hares that have a fur coloration that deviates from what is considered normal by the hunters. Conspicuous fur coloration has been reported in both species and physiological plasticity is a general characteristic among hares (Flux & Angerman 1990).

MtDNA transferred to brown hares

The discrepancy in distribution of mtDNA haplotypes transferred to brown hares when compared to those of sympatric mountain hares call for rejection of the hypothesis of recent and/or current introgression. Current hybridisation between released, captive reared, mountain hares with mtDNA different from the wild mountain and semi-natural brown hares may mediate mtDNA transmission. However, the released hares often

stem from northern Sweden and, thus, the transferred mtDNA should match the mtDNA of mountain hares from northern Sweden, which was not the case. Possibly, introgression of mountain hare DNA pre-dated the introduction of brown hares to Sweden. Although we cannot rule out that some of the unique mtDNA haplotypes were brought to Sweden along with introduced brown hare specimens, there are no signs of introgression of mountain hare mtDNA among brown hares from their native range (cf. Hartl *et al*. 1993, Pierpaoli *et al*. 1999). Also, the most divergent of the transferred lineages (group II in Fig. 2) show no similarity to any particular mountain hare mtDNA lineage when included in a phylogenetic study of European mountain hare subspecies (*see* Sw1, i.e. haplotype 8, in Thulin *et al*. 1997b).

We believe that the observed discrepancy in the distribution of mtDNA haplotypes is due to the extinction, or shifted frequencies, of some of the transferred mtDNA haplotypes after the interspecies transfer. Here follows a potential scenario: During the period of initial range expansion of brown hares right after introduction (e.g. Lönnberg 1908), some brown hare males encountered, courted and bred with mountain hare females. The mtDNA haplotypes of the hybridising mountain hare females were not passed on to future generations of mountain hares because the hybrid females preferred to mate with brown hare males. Therefore, through hybridisation, some mtDNA haplotypes may have become extinct or decreased in frequency among the mountain hares. However, as specimens in the front of expansion usually contribute extensively to newly founded populations (Hewitt 1993), the surviving mtDNA lineages transferred to brown hares were not only preserved, but also reached relatively high frequencies.

The failure to detect signs of hybridisation between the two hare species in continental Europe (Hartl *et al*. 1993, Pierpaoli *et al*. 1999) and in Finland (Schröder *et al*. 1987) as opposed to Sweden (Thulin *et al*. 1997a, Thulin & Tegelström 2002), may be explained by differences in the colonisation history of the brown hares. The brown hare supposedly colonised continental Europe in association with the spread of agriculture (cf. Thulin 2003) and Finland naturally during the last two centuries (Thenius 1980). On the other hand, brown hares were introduced to Sweden by man, which gave the native mountain hares less time to gradually adapt to the alien species. Thus, we believe that these two species of hares provide excellent opportunities for comparisons between antropogenically created hybrid zones (e.g. Sweden) and natural contact zones (e.g. Finland) in similar habitats.

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